**1** **Transcriptional activation of cholesterol metabolism in *BRAF[V600E]*-driven**

**2** **serrated colorectal neoplasia.**

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**16** **Running title:** Cholesterol biosynthesis contributes to intestinal hyperplasia in

**17** *BRAF[V600E]* mutant colorectal cancers

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24 **Abstract**

25 *BRAF* mutations occur early in serrated colorectal cancers, but their long-term

26 influence on tissue homeostasis are poorly characterized. We investigated the impact

27 of short-term (3 days) and long-term (6 months) expression of $Braf^{V600E}$ in the intestinal

28 tissue of an inducible mouse model. We show that $Braf^{V600E}$ perturbs the homeostasis

29 of intestinal epithelial cells, with impaired differentiation of enterocytes emerging after

30 prolonged expression of the oncogene. Moreover, $Braf^{V600E}$ leads to a persistent

31 transcriptional reprogramming with enrichment of numerous gene signatures

32 indicative of proliferation and tumorigenesis, and signatures suggestive of metabolic

33 rewiring. We focused on the top-ranking cholesterol biosynthesis signature and

34 confirmed its increased expression in human serrated lesions. Functionally, the

35 cholesterol lowering drug atorvastatin prevents the establishment of intestinal crypt

36 hyperplasia in $Braf^{V600E}$-mutant mice. Overall, our work unveils the long-term impact

37 of $Braf^{V600E}$ expression in intestinal tissue and suggests that colorectal cancers with

38 mutations in *BRAF* might be prevented by statins.

**Introduction**

The single-layered intestinal epithelium contains functionally distinct cellular populations. Proliferating intestinal stem cells (ISCs), whose self-renewal depends on active Wnt/β-catenin signaling[1], are interspersed among Paneth cells at the bottom of epithelial invaginations, known as crypts of Lieberkühn, and can be identified by selective markers such as LGR5 and OLFM4[2,3]. ISCs generate transient amplifying (TA) cells, which have limited proliferative capability and migrate upwards towards intestinal villi where they differentiate into the absorptive enterocyte lineage or the secretory lineage, which includes Paneth cells, enteroendocrine cells (EECs) and goblet cells.

Colorectal cancer (CRC) is a heterogeneous disease arising from the intestinal epithelium through two main routes[4,5]. Firstly, tumors originating from tubular, villous or tubulovillous precursor adenomas (ADs) localize to the left colon, are characterized by sustained Wnt/β-catenin signaling and are thought to develop from ISCs through a bottom-up fashion[6-9]. Secondly, tumors located in the right colon arise from precursor sessile serrated lesions (SSLs), characterized by a saw-tooth-shaped folding of the dysplastic epithelium, a flat sessile morphology and a mucinous histology[10]. This subtype of CRC develops in a top-bottom fashion though transdifferentiation of committed epithelial cells to gastric metaplasia and enrichment in fetal markers[6,7,11]. The most common driver mutation of SSLs is an activating mutation of the *BRAF* oncogene[12-14]. A valine to glutamate substitution at codon 600 (V600E) generates a constitutive active mutant (BRAF[V600E]*)* serine-threonine kinase that is responsible for the activation of the downstream MEK/ERK arm of the MAPK pathway[15]. Additional molecular features associated with right-sided colonic neoplasia include CpG island methylator phenotype (CIMP), microsatellite instability (MSI) and loss of TGF signaling

64    [10-12,14,16]. Recently, heterogeneity of *BRAF*-driven CRCs has been uncovered through

65    bulk RNA sequencing and two main *BRAF^V600E* CRC subtypes have been identified

66    based on gene expression profile[17]. The BM1 subtype displays a gene expression

67    profile enriched in EMT-related processes, *KRAS* signaling and immune response,

68    whereas the BM2 subtype is enriched in cell-cycle and cycle checkpoints-related

69    processes, such as target genes of the E2F transcription factors and genes involved

70    in the G2 to M transition of the cell cycle.

71          *Braf^V600E* has been reported to be a poor oncogene when expressed in the

72    mouse intestinal epithelium, whereby few tumors develops after long latency[18-20]. This

73    prolonged latency agrees with the knowledge that human CRC develops covertly over

74    10-15 years[21] and indicates that intestinal tissue harbors the oncogenic *BRAF*

75    mutation for a considerable time despite the absence of any clinical manifestation of

76    the disease. Notwithstanding, little is known about the long-term impact of *BRAF^V600E*

77    expression on intestinal homeostasis, and the mechanisms that enable *BRAF*-driven

78    CRC development remain incompletely understood. Recently, the limited intestinal

79    tumorigenesis of *Braf^V600E* mutant mice has been attributed to an imbalance between

80    stemness and differentiation, whereby expression of *Braf^V600E* promotes differentiation

81    of intestinal epithelial cells at the expense of ISCs[18,22].

82          The overall aim of the work described here was to attain a deeper

83    understanding of serrated tumorigenesis and ascertain the impact of long-term

84    expression of *Braf^V600E*. To this end, we used a mouse model carrying an inducible

85    *Braf^V600E* allele[20] targeted to the intestinal epithelium, and performed transcriptional

86    profiling over a 6-month time course. Together with dynamic changes in tissue

87    homeostasis, we show that *Braf^V600E* orchestrates a rapid and persistent transcriptional

88    reprogramming characterized by the enrichment of numerous gene signatures

4

89   associated with *Braf*-driven CRC and additional signatures suggestive of metabolic

90   rewiring. In particular, we observed a robust increase in the expression of cholesterol

91   biosynthesis genes. The functional relevance of those adaptations was confirmed by

92   the ability of atorvastatin, a commonly prescribed pharmacological inhibitor of the

93   mevalonate pathway and cholesterol biosynthesis, to prevent the establishment of

94   crypt hyperplasia in *Braf*^V600E^-mutant mice.  Finally, through the analysis of human bulk

95   and single-cell transcriptomic datasets, we confirmed that an increased cholesterol

96   gene signature is a hallmark of serrated CRC.

97   **Results**

98   **Activation of *Braf*^V600E^ in the intestine leads to crypt hyperplasia and persistence**

99   **of crypt-resident ISCs.**

100      Mice carrying an inducible *Braf*^V600E^ knock-in allele were crossed with *Villin-*

101   *Cre*^ER^ mice to generate *VillinCre*^ER/0^*/Braf*^V600E-LSL/+^ (BVE) and control *VillinCre*^ER^*/Braf*^+/+^

102   (WT) mice. Intraperitoneal injection of tamoxifen in BVE mice enabled expression of

103   the mutant *Braf*^V600E^ allele. Mutant BVE mice displayed reduced survival (median

104   survival 414 days versus 622 days in WT mice) (Supplemental Figure 1A) and, as

105   previously reported, neoplasia developed after long latency and with limited

106   penetrance (Supplemental Figure 1B and C)[18-20]. To gain insight into short- and long-

107   term outcomes of oncogene expression, we performed tissue histology (3 days, 6

108   weeks and 6 months after induction of *Braf*^V600E^) and transcriptomic analysis (3 days

109   and 6 months after induction) of intestinal tissue of BVE and control mice (Figure 1A

110   and Supplemental Figure 2). Analysis of transcriptomic data using GSEA confirmed a

111   robust expression of a MAPK gene signature in mutant mice (Supplemental Figure 3).

112    Firstly, we assessed whether expression of mutant *BRAF* alters homeostasis

113  of the intestinal crypt. Cell count indicated that as early as 3 days post induction,

114  mutant crypts were hyperplastic (Figure 1B). Hyperplasia was linked to increased cell

115  proliferation (Figure 1C) and was persistent, with a significantly increased numbers of

116  cells detected in intestinal crypts of aged mutant mice (Figure 1D). Previous reports

117  have suggested that expression of *Braf^V600E* causes TA cell generation and cellular

118  differentiation at the expense of the ISC population[18,22]. Using the stem cells marker

119  Olfm4[2], we were able to identify a persistent Olfm4+ population localized at the bottom

120  of the intestinal crypt in tissue specimens collected 3 days, 6 weeks and 6 months

121  after induction (Figure 1E). Moreover, stem cell marker genes *Lgr5* and *Olfm4* in BVE

122  mice were expressed at levels comparable to those in control mice 6 months after

123  *Braf^V600E* expression (Supplemental Figure 4A). We also interrogated transcriptomic

124  data, performing GSEA of an ISC signature. This analysis showed a mild

125  downregulation early after *Braf* activation, but this was reverted in 6 month tissues

126  (Supplemental Figure 4B).

127    However, whereas in WT mice ISCs were interspersed with Paneth cells, in

128  long-term induced mice ISCs clustered at the bottom of the intestinal crypt in long-

129  term induced mice (Figure 1D, insets), following a generalized loss and delocalization

130  of Paneth cells (Supplemental Figure 5).  Finally, in keeping with the role of *Braf^V600E*

131  in serrated neoplasia, villi from BVE mice developed a saw-toothed appearance early

132  after oncogene expression (Supplemental Figure 6A) and, consistent with the

133  establishment of tissue hyperplasia (see below), villi length was significantly increased

134  in 6 weeks and 6 months mutant mice (Supplemental Figure 6B).

135  **Expression of *Braf^V600E* modifies the abundance and distribution of**

136  **differentiated intestinal populations**

137    We then assessed the impact of mutant *Braf* on differentiated intestinal cell

138    populations. As early as 6 weeks post induction of *BRAF^V600E^*, the intestinal crypts

139    were deprived of Paneth cells, which migrated upwards towards the villi (Supplemental

140    Figures 5) and were progressively lost, a phenotype previously observed in mouse

141    models with altered MAPK activity[23].

142    In addition, GSEA revealed an initial increase in the expression of an intestinal

143    differentiation gene signature 3 days post *Braf^V600E^* induction. However, unexpectedly,

144    the same signature was downregulated following long-term expression of mutant *Braf*

145    (Figure 2A). At this stage, we also identified a significant downregulation of an

146    intestinal enterocyte signature, which was reflected by reduced Alpl histological

147    staining (Figure 2B and C). A late impairment in the differentiation of enterocytes was

148    confirmed by TEM, which showed shortened and less abundant microvilli on the

149    surface of mutant enterocytes (Figure 2D).

150    Next, we visualized and quantified mucus-producing goblet cells and EECs

151    using Alcian Blue staining and immunofluorescence staining for Chromogranin A

152    (ChrA), respectively (Supplemental Figure 7). Quantification of Alcian Blue- and ChrA-

153    positive cells revealed an increased number of goblet cells, whereas EECs were

154    reduced after long-term (6 months) expression of *Braf^V600E^* (Figure 2E). rt-qPCR

155    analysis confirmed the reduced expression of ChrA (Figure 2F).

156    These data suggest that *Braf^V600E^* alters the balance of intestinal secretory and

157    absorptive cell lineages. The analysis of the long term impact of oncogene expression

158    particularly unveiled a significant impact on the numbers of goblet cells and EECs, and

159    a broader downregulation of gene signatures associated with intestinal differentiation

160    and, more specifically, with enterocyte differentiation.

161        Overall, our data confirm previous evidence of reduced stemness and

162    increased differentiation early after induction of *Braf^(V600E)*. They show, in addition, that

163    those changes are transient and that stemness is restored in the intestinal tissue at

164    the expense of differentiation. These findings emphasize the importance of long-term

165    analysis of oncogene expression in the intestinal tissue and they are congruent with

166    recent data suggesting a top-down origin of human serrated neoplasia triggered by

167    dedifferentiation of intestinal cells[6].

168    **_Braf^(V600E)_ expression regulates a transcriptional program linked to CRC**

169        Next, we identified the molecular pathways regulated by *Braf^(V600E)* by

170    interrogating the Molecular Signatures Database (MSigDB)[24] (Supplemental Figure 8).

171    Signatures that scored high included pathways associated with cellular proliferation

172    and the BM2 subtype of human CRC, such as Myc, E2F targets and G2/M checkpoint

173    signatures (Figure 3A), which were significantly enriched as early as 3 days post

174    *Braf^(V600E)* induction. The same signatures were confirmed to be enriched in *BRAF^(V600E)*

175    mutant CRCs compared with normal colon tissue from the TCGA dataset[8]

176    (Supplemental Figure 9). We also observed an enrichment of a canonical Wnt pathway

177    signature in the mouse mutant intestinal tissue (Figure 3B), in agreement with previous

178    findings indicating that activation of *Braf^(V600E)* results primarily in intestinal tumors

179    displaying Wnt-pathway activation[19,20,22]. More tellingly, long-term activation of

180    *Braf^(V600E)* was associated with the expression of a *BRAF*-mutant CRC signatures that

181    predicts poor prognosis in CRC patients[25] (Figure 3C).

182        To ascertain whether MAPK activity was necessary for the establishment of the

183    observed transcriptional changes, we treated mice with the MEK inhibitor PD184352

184    (MEKi). Mice received daily treatments with vehicle or MEKi for 3 consecutive days

185 and were then culled for transcriptomic analysis (Supplemental Figure 10A). MEKi

186 administration achieved a marked decrease in phospho-Erk in intestinal tissue, as

187 assessed by Western blot analysis (Supplemental Figure 10B), and a stark repression

188 of the MAPK signature[19] (Supplemental Figure 10C). Notably, pharmacological

189 inhibition of the MAPK pathway reverted the enrichment of some, but not all,

190 signatures. In particular, the Myc signature, the canonical Wnt signature and the

191 Popovici poor-prognosis signature all depended on a functional MAPK pathway

192 (Figure 3D).

193      The origin of *BRAF^V600E* CRC remains elusive. Recent evidence suggests that

194 serrated neoplasia arises through an intermediate gastric metaplasia and a reversion

195 to an embryonic cellular stage, sustained by a fetal gene signature[6,7,11]. Indeed,

196 serrated lesions show enrichment for gene expression associated with metaplasia and

197 fetal genes (Supplemental Figure 11A). In agreement with previous observations[11],

198 expression of *Braf^V600E* triggers significant and persistent enrichment of the fetal

199 signature in intestinal tissue, which we show to be dependent on MAPK signaling

200 (Supplemental Figure 11B). However, we did not detect significant changes in the

201 metaplasia gene signature[6], although two genes within the signature, namely

202 Aquaporin 5 (*Aqp5*) and Annexin A10 (*Anxa10*), were consistently upregulated in

203 mutant tissue in a MAPK-dependent fashion (Supplemental Figure 12). Interestingly,

204 *Anxa10* has been previously reported to be a specific marker for human SSL and

205 CRCs of the serrated pathway[22,26,27].

206      Overall, these data indicate that activation of *Braf^V600E* orchestrates changes in

207 the intestinal transcriptome that reflect the transcriptional reprogramming underlying

208 colorectal carcinogenesis. These changes are durable, persisting for up to 6 months

209 following oncogene expression.

***Braf^V600E* expression rewires gene signatures of cholesterol metabolism to drive crypt hyperplasia**

We noticed that the top scoring gene signature enriched in *BRAF*-mutant mice was a cholesterol signature at both 3 days and 6 months following *Braf^V600E* induction (Figure 4A). The robust increase in this signature was also MAPK-dependent (Figure 4B). The leading-edge genes responsible for the signature enrichment included several key metabolic enzymes of the mevalonate pathway, such as *Idi1*, *Mvk*, *Fdft1*, *Fdps*, *Sqle*, *Hmgcs1*, *Mvd*, *Hmgcs2.* Increased expression of cholesterol biosynthesis genes was also confirmed by rt-qPCR on RNA extracted from intestinal tissue (Figure 4C). Next we confirmed enrichment of the cholesterol biosynthesis signatures in RNA-seq datasets from two independent mouse models of *BRAF*-driven CRC (Figure 4D)[11,18].

Cholesterol biosynthesis contributes to tumorigenesis and can be inhibited using statins, a widely prescribe category of drugs that target the mevalonate pathway rate-limiting enzyme HMG-CoA reductase. Several studies indicate that regular statin use reduces the incidence of several malignancies, including CRC[28,29]. Hence, we reasoned that cholesterol biosynthesis contributes to the establishment of the durable crypt hyperplasia observed following induction of *Braf^V600E*and its inhibition by statins would prevent the formation of hyperplastic crypts. To test this hypothesis, we treated mice with daily doses of atorvastatin starting one week before induction of *Braf^V600E* and collected tissue 3 days post tamoxifen administration. Counting of crypt epithelial cells confirmed that statin treatment prevented the establishment of crypt hyperplasia in mutant intestinal tissue (Figure 4E). Mechanistically, atorvastatin did not alter crypt proliferation (Figure 4F), but significantly increased apoptosis assessed through cleaved PARP immunohistochemistry (Figure 4G).

235    Overall, these data indicate that *Braf^V600E* elicits a transcriptional

236    reprogramming of cellular metabolism indicative of increased cholesterol biosynthesis,

237    which contributes to the establishment of tissue hyperplasia by increasing survival of

238    crypt cells. The results suggest that statins might prevent colorectal cancer harboring

239    mutant *BRAF^V600E*.

240    **Expression of *BRAF^V600E* establishes a network of transcription factors that**

241    **mediates the enrichment in the cholesterol biosynthesis gene signature in**

242    **human SSLs**

243    Next, we investigated whether the enrichment of the cholesterol metabolism

244    gene signature induced by *Braf^V600E* in the mouse intestine was preserved in human

245    SSLs. To this end, we analyzed three independent datasets of SSLs, which enabled

246    comparison between SSLs, traditional ADs and normal right colon tissue[30-32]. These

247    analyses showed a robust enrichment in the cholesterol biosynthesis signature when

248    SSLs were compared to either normal tissue or ADs (Figure 5A). Notably, no

249    significant enrichment was observed when ADs were compared to normal tissue

250    control, suggesting that the transcriptional rewiring of cholesterol metabolism is

251    specific to SSL (Supplemental Figure 13A). Since bulk transcriptomic data cannot

252    distinguish between the contributions by tumor or non-tumor cells to signatures'

253    enrichment, we ascertained expression of the cholesterol metabolism signature in

254    transformed epithelial cells, by querying a scRNAseq dataset of SSLs and normal

255    tissue (NL)[6]. Clustering of the SSL and NL single cells revealed six canonical cell types

256    and one serrated specific cell (SSC) subtype[6] (Figure 5C and Supplemental Figure

257    14A). Notably, when the expression of the cholesterol biosynthesis signatures were

258    scored in the cell clusters using the UCell tool[33], SSCs exhibited significantly higher

259    expression of the signature, compared with other cell types (Figure 5D-E). Key

260   metabolic enzymes of the mevalonate pathway were expressed in SSCs, some of

261   them showing a significant enrichment compared to non-cancerous cells

262   (Supplemental Figure 14B).

263   These data indicate that the transcriptional metabolic rewiring of cholesterol

264   metabolism observed in *Braf^V600E* mouse intestinal tissue recapitulates comparable

265   adaptations of human colorectal lesions and is a feature of serrated lesions.

266   To investigate how *BRAF^V600E* increases transcription of the cholesterol gene

267   signature in human SSLs, we first applied the SCENIC method[34] to infer transcription

268   factors and gene regulatory networks from scRNA-seq data of SSLs and NL. By doing

269   so, we identified regulons that are specific to annotated cell types based on RSS[35]

270   (Supplementary Figure 15). Next, we employed iRegulon[36] to pinpoint which

271   transcription factors active in SSCs were predicted to regulate the differentially

272   expressed genes within the cholesterol gene signature (cut off ≥5 target genes). We

273   singled out six transcription factors, namely *CEBP*, *CREB3*, *NR2F1*, *KLF16*, *SP6* and

274   *FOSL1*, whose regulons are strongly enriched in SSCs (Figure 6A) and which

275   contribute towards the establishment of the cholesterol metabolism signature in SSCs

276   (Figure 6B).

277   **Discussion**

278   Ten percent of human CRCs, which originate from serrated lesions, harbor the

279   V600E mutation in the *BRAF* oncogene[13]. This mutation is thought to contribute to

280   tumorigenesis through the activation of the MEK/ERK arm of the MAPK pathway. Yet,

281   despite the development of CRC being a decade long process, the long-term impact

282   of oncogene expression on the intestinal epithelium has not been investigated. Here,

283   for the first time, we assessed this issue using a genetically modified mouse model,

284  the *VillinCre^ER/0^/Braf^V600E-LSL/+^* (BVE), which enables tamoxifen-inducible expression of

285  mutant *Braf^V600E^* in the intestinal epithelium. We used a combination of transcriptomic

286  and histological analyses to compare short-term and long-term impacts of *Braf^V600E^*

287  expression. By doing so, we were able to identify changes in intestinal epithelial

288  populations that were evident only after prolonged oncogene expression. Interestingly,

289  despite failing to observe any loss of ISCs as reported by others[18,22], we did find

290  downregulation of the ISC signature early after *Braf^V600E^* induction. However, ISC

291  signature was recovered and downregulation reverted after 6 months. Although it is

292  currently unclear what enables this adaptation in the BVE mice, these changes could

293  be explained by the well-established plasticity of the intestinal epithelial cells[37]. A

294  striking alteration of Paneth cells was evident in 6-week and 6-month induced animals.

295  Paneth cells lost their localization at the base of the intestinal crypt and migrated

296  towards the villi, where they displayed a rounded morphology and were similar to the

297  intermediary cells expressing both Goblet and Paneth cell markers described by

298  Riemer and colleagues[22]. A similar phenotype was reported in other mouse models

299  with intestinal expression of *Braf^V600E^* and a MAPK-dependent loss of Paneth cells was

300  also described in models of mutant *Kras*[18,23,38]. Consistent with these observations,

301  analysis of intestinal tissue after long-term *Braf^V600E^* activation revealed an impact on

302  other secretory lineages. We observed changes in numbers of both Goblet cells and

303  EECs. However, it should be noted that this reduction does not reflect the total number

304  of cells. Indeed, since villi length was increased in mutant mice, cell numbers were

305  normalized to villi length. Notwithstanding, reduction in RNA levels of *ChrA* confirmed

306  a general reduction in EECs. Whereas the increase in goblet cells probably reflects

307  the mucinous nature of serrated neoplasia, the changes in EECs are particularly

308  intriguing. Indeed, human *BRAF^V600E^* CRCs accumulate EEC progenitor cells that fail

309  to complete their differentiation. These progenitor EECs secrete the TFF3 protein, a

310  member of the Trefoil family, which supports CRC progression through activation of

311  the PI3K/AKT pathway[39]. These data warrant additional research into the impact of

312  *BRAF* mutations on the differentiation and activity of EECs in the context of serrated

313  neoplasia.

314      Transcriptomic analysis through GSEA confirmed a negative enrichment of an

315  intestinal differentiation signature after prolonged *Braf*[V600E] expression. This was likely

316  driven by a significant downregulation of an enterocyte differentiation signature, which,

317  to the best of our knowledge, has not been reported previously. The possible reduction

318  in enterocytes was confirmed by reduced Alpl staining in intestinal tissue. Overall, our

319  data suggest that the long-term impact of *BRAF*[V600E] is associated with a reduction in

320  tissue differentiation, perhaps necessary to rebalance the initial decrease in

321  stemness[18,22]. These data are in agreement with published evidence indicating that

322  *BRAF*[V600E] suppresses hallmarks of intestinal differentiation, which can be restored

323  upon pharmacological inhibition of the MAPK pathway[40].

324      The activation of *Braf*[V600E] led to a rapid and persistent enrichment in MAPK-

325  dependent gene signatures associated with proliferation and CRC progression,

326  including a fetal-like gene signature, a Myc signature, a G2/M signature and an E2F

327  target gene signature. The latter two have been described as significant features of

328  the BM2 molecular subtype of CRC[17]. We also observed the induction of a Wnt

329  signaling related signature, which was promptly downregulated by inhibition of the

330  MAPK pathway. The association between Wnt pathway and *BRAF*-driven CRC is still

331  unclear. In mice, Rad and colleagues observed a progressive increase in Wnt

332  activation during neoplasia progression[19], which was associated with nonsense or

333  frameshift mutations in the *Apc* gene and activating mutations in the *Ctnnb1* gene,

334    which encodes β-catenin. Mutations affecting Wnt-related genes have also been

335    reported in human serrated CRCs. Truncating mutations of the Wnt negative regulator

336    gene *RNF43* are common in MSI CRCs[41], and non-truncating mutations in the *APC*

337    gene have also been detected[42]. However, the contribution of Wnt signaling to MSI

338    CRCs remains controversial with some studies reporting lack of aberrant nuclear β-

339    catenin localization[43], a surrogate marker for Wnt pathway activation, but others

340    showing significant nuclear accumulation in more advanced serrated lesions[42,44].

341    Notwithstanding, GSEA in BVE mice indicates that, despite the lack of neoplastic

342    lesions, expression of *Braf^V600E* triggers a transcriptional reprogramming congruent

343    with tumorigenesis.

344        Metabolic rewiring is a common hallmark of cancer, including CRC[45-47] and we

345    have recently, reported on the role of amino acid metabolism in CRC progression[48,49].

346    Here, we provide novel data that strongly support the establishment of a

347    transcriptionally-driven metabolic reprogramming of cholesterol biosynthesis following

348    activation of *Braf^V600E*. We have also confirmed similar changes in human datasets of

349    SSLs, suggesting that this metabolic adaptation is a feature of serrated neoplasia.

350    Using bioinformatics tools SCENIC and iRegulon, we have finally identified the chief

351    transcription factors that are responsible for the transcriptional rewiring: *CEBP*,

352    *CREB3*, *NR2F1*, *KLF16*, *SP6* and *FOSL1*. The transcription factor CREB3 has been

353    linked to regulation of cholesterol in response to the unfolded protein response[50].

354    Similarly, the orphan nuclear receptors NR2F1 and 2 (also known as chicken

355    ovalbumin upstream-promoter transcription factor, COUP-TFI and II) have been

356    reported to regulate genes associated with cholesterol metabolism[51-53]. A role for

357    FOSL1 in promoting cholangiosarcoma growth through regulation of HMG-CS1 along

358    the mevalonate pathway has also been described[54]. Our data warrant further

359    investigation to dissect the contribution of these transcription factors to the regulation

360    of metabolic rewiring in *BRAF* mutant CRCs and details of their regulation by MAPK.

361    Importantly, statins have been suggested to possess cancer preventive properties, but

362    the association between the occurrence of adenoma or CRC and exposure to statins

363    is controversial[28,55-57]. Very little is known about whether protection by statins depends

364    on the specific molecular makeup of the tumor[58]. The sensitivity of *BRAF* mutant CRC

365    cell lines to statin treatment has been suggested to depend on BMP signaling through

366    a functional *SMAD4* gene[59]. BMP signaling belongs to the TGF-beta superfamily and

367    mutations in the TGF-beta pathway are common in *BRAF*-mutant CRCs. Whether this

368    affects sensitivity to statins remains to be ascertained. Nonetheless, we observed a

369    similar behavior in the response of *BRAF*-mutant CRC cells to atorvastatin *in vitro*:

370    RKO cells with intact *SMAD4* showed sensitivity to statin treatment, whereas HT29

371    cell harboring homozygous mutations in *SMAD4* were resistant (Supplemental Figure

372    16). These observations are in agreement with the evidence provided here that

373    atorvastatin prevents crypt hyperplasia in the *Braf*-mutant intestine of BVE mice, which

374    harbor a functional BMP pathway.

375          Overall, our data show that one of the most significant and consistent effects of

376    *BRAF^V600E* expression in mouse tissue and human serrated lesions is the enrichment

377    of a gene expression signature of cholesterol biosynthesis that contributes to the

378    establishment of epithelial hyperplasia. These findings suggest that the incidence of

379    *BRAF*-mutant CRC could be reduced using statins and warrant further targeted

380    investigations into the specific statin sensitivity of this subtype of CRC.

381    **Materials and Methods**

382    **Cell lines and MTT assay**

383      HT29 CRC cells were grown in DMEM supplemented with 10% foetal calf

384    serum (FCS) and Glutamax. RKO CRC cells were grown in MEM supplemented with

385    10% FCS and Glutamax. Cells were incubated at 37°C and 5% $CO_2$. For MTT assay,

386    500 to 2000 cells were plated in 96-well plates. A 50 mM atorvastatin in DMSO stock

387    solution was diluted in media (supplemented with dialyzed serum) to concentrations

388    of 1, 5 and 10 μM. Three 96-well plates were run for each assay, each treated for

389    either 24, 48 or 72 hrs. Cells were then incubated with MTT (2.5 mg/ml) at 37°C 2 hrs.

390    To dissolve the formazan produced by the metabolism of MTT, 50 μl of DMSO was

391    added to each well. The plates were then incubated for 30 minutes on a shaker before

392    the optical density was read using a FluoStar OPTIMA plate reader (BMG-Labtech

393    Ltd, UK).

**Mouse colony and genotyping**

395      All mice were C57BL6/j background. All animal experiments were performed

396    according to Home Office guidelines under project licenses (PPL) PC4E1710A and

397    P7B8067BB. Mice were housed in the pathogen-free Preclinical Research Facility

398    (PRF) at the University of Leicester and were fed ad libitum with AIN93 diet (TestDiet,

399    USA, Cat#5801-G), under a climate-controlled environment with 12hr day/night cycle.

400    For transgene induction, 8-12 week-old double heterozygous *VillinCre^{ER/0}/Braf^{V600E-}*

401    *^{LSL/+}* mice received intraperitoneal injections of 1 mg tamoxifen on five consecutive

402    days at 24hr intervals, unless otherwise stated. Tamoxifen solution was prepared as

403    a 10 mg/ml stock solution solubilized in corn oil. Control mice were *VillinCre^{ER/0}/Braf^{+/+}*

404    which received tamoxifen, or *VillinCre^{ER/0}/Braf^{V600E-LSL/+}* treated with vehicle. After

405    euthanasia, the small intestine was flushed with PBS and cut into 6 sections rolled to

406    create a "Swiss roll" and fixed in 4% [w/v] paraformaldehyde on a shaker overnight at

407    room temperature. Fixed tissues were placed in embedding cassettes and submerged

17

408  in 70% ethanol and processed by the Core Biotechnology Services (CBS) histology

409  facility, University of Leicester. Small pieces of tissue (~0.5 cm) were snap frozen in

410  liquid nitrogen for protein extraction or snap frozen in RNAlater solution (Sigma) for

411  RNA extraction.

412      DNA from mouse ear snips was extracted using the DNeasy Blood & Tissue Kit

413  (QIAGEN) according to the manufacturer's instructions. PCR reaction mix for the *Braf*

414  locus was: 10.5 µL of template DNA, OCP 125 (FWD primer) 1 µL (10 pmol), OCP

415  137 (REV-HET primer), 0.5 µL (5 pmol) OCP 143 (REV-WT primer) 0.5µL (5 pmol),

416  MyTaq Red (2x) (Meridian Bioscince BIO-25043) 12.5 µL. The PCR program was:

417  95°C for 2 min, 30 cycles of 94°C for 15 seconds, 60°C for 15 seconds, 72°C for 15

418  seconds; 5 min at 72°C. For Cre$^{ER}$ genotyping the PCR mix was: 10.5 µL of template

419  DNA, OCP 361 (FWD primer) 1 µL (10 pmol), OCP 362 (REV primer) 1 µL (10 pmol),

420  MyTaq Red (2x) (Meridian Bioscince BIO-25043) 12.5 µL. The PCR program was:

421  95°C for 4 min, 30 cycles of 94°C for 20 seconds, 57°C for 20 seconds, 72°C for 25

422  seconds; 5 min at 72°C.  Primers (Sigma) sequences are listed in Supplemental table

423  1. The PCR products were visualized using 1.8% agarose gel with ethidium bromide

424  (0.5 µg/mL) and imaged using the Syngene G:BOX Chemi XR5. Gels were examined

425  for the presence of PCR bands at 140bp (*Braf$^{LSL-V600E}$* allele) and 466bp (wild-type *Braf*

426  allele) and 300 bp (*Villin-CreERT* allele).

### Statin treatment *in vivo*

428      Thirteen-week-old female *VillinCre$^{ER/0}$/Braf$^{V600E-LSL/+}$* mice received either 10

429  mg/kg atorvastatin by oral gavage or 5% DMSO/PBS vehicle control daily for 12 days.

430  A 20 mg/mL atorvastatin (Generon) stock in DMSO was freshly diluted to a 1 mg/mL

431  working solution in PBS. Days 1-7 of the study were a daily pre-treatment period of

432    atorvastatin/vehicle. Days 8-10 consisted of both atorvastatin/vehicle gavage and

433    intraperitoneal tamoxifen injection to induce *Braf^{V600E}* expression. On the final day of

434    the study at 3-days post- *Braf^{V600E}* induction, mice received intraperitoneal injections

435    of 200 µL of 20 mM BrdU solution (Sigma) 3 hrs before tissue harvesting.

436    **Histology**

437    Tissue sections (4 µm) and H&E staining were performed by the Histology

438    Facility, Core Biotechnological Services, University of Leicester.

439    Immunohistochemistry was performed using the Novolink Polymer Detection Systems

440    kit (Leica Biosystems), according to manufacturer's instructions. SuperFrost Plus™

441    Adhesion slides (Fisher Scientific) containing FFPE tissue sections were incubated at

442    65°C for 30 minutes. Tissues were dewaxed and rehydrated through 3-minute serial

443    immersions in xylene, 99% industrial methylated spirit (IMS), and 95% IMS twice each,

444    followed by 5 minutes in running tap water. Following antigen retrieval (Supplemental

445    Table 2), slides were incubated for 5 minutes in peroxidase block, washed twice in

446    PBS, incubated for 5 minutes in the protein block solution and washed twice in PBS.

447    Tissues were incubated with primary antibodies (Supplemental table 2) (3% BSA/PBS)

448    overnight at 4°C. After incubation, sections were incubated in Polymer solution for 30

449    minutes, washed in PBS, and incubated in DAB solution for 5 minutes. Tissues were

450    counterstained in Mayer's hematoxylin for 5 minutes and subsequently washed in tap

451    water. Tissues were dehydrated through 3-minute serial immersions in 95% IMS twice,

452    99% IMS twice, xylene twice. Slides were then mounted onto glass coverslips using

453    DPX (Sigma Aldrich). Whole slide images were acquired using the Nanozoomer XR

454    digital slide scanner (Hamamatsu Photonics) and visualized using NDP.view2

455    software. For immunofluorescence (IF), antigen-retrieved tissues were blocked in 5%

456    BSA/0.3% Triton X-100 in PBS for 30 minutes, washed with 1% BSA/0.3% Triton X-

457    100 in PBS twice for 10 minutes each and incubated with SP-1 Chromogranin A

458    (ChgA) primary antibody (ImmunoStar) overnight at 4°C. Slides were washed the next

459    day in wash buffer solution twice for 10 minutes each. Alexa Fluor 568 secondary

460    antibody (Invitrogen) was incubated for 1 hr at RT in darkness. Slides were washed

461    and sealed with coverslips using ProLong™ Glass Antifade Mountant containing DAPI

462    (Invitrogen™). IF images were captured using the Vectra® Polaris™ imaging system

463    (Akoya Biosciences), and images were viewed using Phenochart software v1.1.0

464    (Akoya Biosciences).

465    Alcian blue staining for visualization of goblet cells was performed using the

466    Alcian Blue (pH 2.5) Stain Kit (Vector Laboratories). Rehydrated slides were heated

467    at 65°C and incubated in 3% acetic acid solution for 3 minutes, followed by incubation

468    with Alcian Blue solution (pH 2.5) for 30 minutes at RT. Sections were rinsed in 3%

469    acetic acid solution, running tap water and distilled water. Sections were then counter-

470    stained with Enhanced Nuclear Fast Red (Vector Laboratories) for 5 minutes, rinsed

471    in running tap water and distilled water, and dehydrated for mounting.

472    Intestinal alkaline phosphatase staining for detection of enterocytes was

473    performed using Vector Red alkaline phosphatase staining kit (Vector Laboratories).

474    Rehydrated slides were incubated in substrate working solution for 30 minutes and

475    washed in running water for 5 minutes. Tissues were counterstained in Mayer's

476    hematoxylin (Leica Biosystems), dehydrated and mounted in DPX.

477    **Electron Microscopy (EM)**

478    All EM reagents were from Agar Scientific, unless otherwise stated. For

479    Transmission Electron Microscope material was fixed overnight at 4°C in 2.5%

480    glutaraldehyde and 4% paraformaldehyde in 0.1M cacodylate buffer, pH7.2 and

481  washed in 0.1M cacodylate buffer. After the secondary fixation in 1% osmium tetroxide

482  and 1.5% potassium ferricyanide (Merck Life Science) at RT, samples were treated

483  with 1% tannic acid (VWR) dehydrated in ethanol series followed by propylene oxide

484  (Merck Life Science) and embedded in Epon 812 (TAAB Laboratory Equipment Ltd).

485  Samples were sectioned to 70 nm thick using a Reichert Ultracut E ultramicrotome,

486  collected onto copper mesh grids and stained for 5 minutes in lead citrate. Samples

487  were viewed on a JEOL JEM-1400 TEM with an accelerating voltage of 120 kV. Digital

488  images were collected with an EMSIS Xarosa digital camera with Radius software.

**Western Blotting**

490  Tissues were homogenized in 400 µl of ice cold RIPA buffer, containing

491  protease inhibitor cocktail, and phosphatase inhibitor cocktail (Roche). The

492  homogenized solution was kept on ice for 10 minutes with vortexing every minute.

493  Samples were then centrifuged for 10 minutes at 12,000 rpm, at 4°C. Supernatants

494  were collected and protein concentration estimated with Lowry assay. Protein lysates

495  were diluted to 1 mg/ml with SDS loading buffer (0.0625 M Tris-HCl pH 6.8, 2% SDS,

496  0.4 ml of 0.025% Bromophenol blue, 20% glycerol, 5% $\beta$-mercaptoethanol) and

497  incubated at 95°C for 10 minutes. 15 $\mu$l of each sample was then run on acrylamide

498  gels (Miniprotean III cell, BioRad) with pre-stained SDS-PAGE protein markers (All

499  Blue Precision Plus Protein Standards, BioRad). Samples were then transferred to

500  nitrocellulose membranes (Syngene) using a semi-dry transfer blot (BioRad) as per

501  manufacturer's instructions. For p-ERK1/2 (Rabbit, Cell Signaling, 9101S),

502  membranes were blocked in 5% BSA (Sigma Aldrich, A3059) in TBST for 90 minutes

503  followed by incubation with primary antibody diluted 1:500 in 5% BSA in TBST

504  overnight at 4°C. For ERK2 (Mouse, Santa Cruz, sc-1647) membranes were blocked

505  in 5% milk in TBST and incubated with the primary antibody diluted 1:1000 in 5% milk

506   in TBST overnight at 4°C. Membranes were washed in TBST, 3 times for 10 min each,

507   and then incubated with secondary antibody (1:5000) at room temperature for 1 hour

508   (anti-Rabbit HRP Sigma A6154, anti-Mouse HRP Sigma A4416) followed by TBST

509   washes 3 times. Bands were visualized using SuperSignal West Pico

510   Chemiluminescent substrate kit (Thermo Fisher Scientific, 34580) and photographic

511   film (Fuji).

**RNA extraction**

512

513   RNA was extracted from intestinal tissue on a Promega Maxwell 16 using the

514   Maxwell 16 LEV simplyRNA Cells Kit (Reference AS1270). RNA quality and quantity

515   was assessed using the 2100 Bioanalyzer Instrument (Agilent).

**Transcriptomic analysis**

516

517   *RNA-sequencing (RNA-seq).* For RNA-seq, four *VillinCre^{ER/0}/Braf^{V600E-LSL/+}* 8-

518   12-week old mice were induced with tamoxifen and four were treated with vehicle to

519   be left uninduced and used as controls to identify differentially expressed (DE)

520   genes. Each experimental group contained two male and two female animals. Mice

521   received intraperitoneal injections of 2 mg tamoxifen on two consecutive days.

522   Intestinal tissue was harvested 6 months post-induction. Indexed RNA libraries were

523   prepared using the NEBNext® Single Cell/Low Input RNA Library Prep Kit for

524   Illumina (NEB ref # E6420S) according to manufacturer's standard protocol and

525   sequenced by 75 bp-paired-end sequencing on Illumina Novaseq, with the aim to

526   obtain approximately 25 million reads per sample.

527   *Microarrays.* For microarrays, 12 mice 8-12-week old were split into two groups

528   of six with an equal number of males and females. Six mice were *VillinCre^{ER/0}* controls

529   and six were *VillinCre^{ER/0}/Braf^{V600E-LSL/+}*. Both groups received three consecutive daily

530    doses of 2 mg tamoxifen and tissue was harvested 3 days after the last injection for

531    short term study and 6 months after for the long-term study. Animals subjected to

532    inhibition of MEK kinase with PD184352 treatment received two injections of 100µl

533    tamoxifen (10mg/ml) on two consecutive days followed by 3 injections of

534    inhibitor/vehicle on three consecutive days. PD184352 (Selleckckem) was prepared

535    in 10% DMSO (Sigma), 10% Cremophor EL (Calbiochem) in water. RNA was

536    extracted as described above and transcriptome profiling was conducted using the

537    SurePrint G3 Mouse Gene Expression v2 8x60K Microarray Kit (Agilent, #G4852B),

538    according to manufacturer's instructions.

539    **Real-time PCR (rt-qPCR)**

540    Reverse transcription of RNA was performed using the High-Capacity RNA-to-

541    cDNA™ Kit (Applied Biosystems™) with a random primer approach. The 2X master

542    mix was prepared according to the manufacturer's instructions, and 2 µg of purified

543    RNA was added per 20 µL reaction. Reactions with and without the reverse

544    transcriptase enzyme were performed in tandem, and samples without the enzyme

545    were used as negative controls in subsequent rt-qPCR assays. The reverse

546    transcription reactions was performed using the program: 25°C for 10 min, 37°C for

547    120 min, 85°C for 5 min.

548    TaqMan® Gene Expression Assays (Fisher Scientific) were used for rt-qPCR

549    reactions (Supplemental Table 3), using the StepOnePlus™ rt-qPCR System (Fisher

550    Scientific) equipped with StepOnePlus™ Software v2.3. The thermocycler program

551    was: 50°C for 2 min, 95°C for 20 sec, then 40 cycles of: 95°C for 1 sec, 60°C for 20

552    sec. Each PCR reaction contained 1 µL of template DNA/control/water, 5 µL of 2X

553    TaqMan Fast Advanced Master Mix (Applied Biosystems) and a TaqMan Gene

554 Expression Assay (Thermo Scientific) containing 900 nM forward and reverse primers

555 and 250 nM probe and 10 µL with nuclease-free water. Each sample was run in

556 technical triplicates. rt-qPCR data were then analyzed using the ΔΔCt method. Ct

557 values were normalized to endogenous control (EC) genes. We employed the

558 NormFinder Excel Add-In, v0.953[60] to assess four candidate genes for use as

559 endogenous controls: glucuronidase beta (*Gusb*), beta-2-microglobulin (*B2m*), beta-

560 actin (*Actb*), and TATA-box binding protein (*Tbp*). The top three genes *Gusb*, *B2m*,

561 *Tbp*) with the most stable expression across experimental groups were selected as

562 EC genes for normalization of RT-qPCR assays in accordance with the MIQE

563 guidelines[61].

**Bioinformatics analysis**

565 Bioinformatics analysis was performed using the statistical computing software

566 R version 4.0.5 and the integrated development environment program RStudio version

567 1.3.1093. For RNA sequencing, FASTQ files sequencing quality was assessed using

568 FastQC (v0.11.9). Sequencing adapter content was trimmed using FastP (v0.20.0)[62].

569 The pre-processed reads were then aligned to the mouse GRCm38/mm10 reference

570 genome using Spliced Transcripts Alignment to a Reference (STAR) (v2.7.3a)[63]. Gene

571 mapping and quantification was performed using DESeq2 (v1.34.0)[64]. For microarray

572 data, the R Bioconductor package Linear Models for Microarray Data (Limma)

573 (v3.50.1) was used to read raw text files from single-channel Agilent RNA microarrays

574 and perform differential expression analysis.

**Gene Set Enrichment Analysis (GSEA)**

576 Agilent gene identifiers (IDs) from microarray analysis and RefSeq gene IDs

577 from RNA sequencing were converted into Entrez IDs using BioMart (v2.50.3)[65] to

24

578     ensure gene IDs were compatible with GSEA functions and The DE genes were

579     ranked using Wald statistic. The R Bioconductor package clusterProfiler[66] was used

580     to perform GSEA and statistical testing using Hallmark gene signatures from the

581     Molecular Signatures Database (MSigDB)[67]. P-values were adjusted for multiple-

582     hypothesis testing using the Benjamini-Hochberg method. Enrichment plots were

583     generated using R Bioconductor package enrich plot (v1.14.2).

584     **Analysis of publicly available datasets**

585     Publicly available datasets (GSE106330[18], GSE168478[11], E-MTAB-6951[32],

586     GSE45270[30] and TCGA-COAD/READ[8]), were obtained from Gene Expression

587     Omnibus     (GEO)     (https://www.ncbi.nlm.nih.gov/geo/),     ArrayExpress

588     (https://www.ebi.ac.uk/arrayexpress/) and The Cancer Genome Atlas (TCGA)

589     (https://www.cancer.gov/tcga). For GSE106330, normalized gene expression matrix

590     was retrieved from GEO. For GSE168478, matrix of raw counts was obtained and

591     further normalized using DESeq2 (v1.36.0) R package. Fastq files were downloaded

592     from ArrayExpress for E-MTAB-6951. Reads, after quality control with fastQC

593     (v0.11.5), were aligned to the human reference genome GRCh38 and quantified using

594     STAR (v2.7.9a) with the following parameters: --quantMode GeneCounts, --

595     outFilterScoreMinOverLread     0.33,     --outFilterMatchNmin     40,     --

596     outFilterMatchNminOverLread 0.33. Count files were loaded into R and normalized

597     using Deseq2 package. Expression CEL files were downloaded from GSE45270 and

598     loaded into R using read.celfiles() function from affy R package (v1.74.0)[68].

599     Background correction normalization and expression estimation was done with rma()

600     function. TCGA-COAD/READ STAR count matrices were accessed using

601     TCGAbiolinks R package (v2.24.3)[69] and normalized with DESeq2 package.

602    GSEA of analyzed public datasets was performed with GSEA software

603    (v4.2.2)[70]. All matrixes, generated as described above, were saved in GCT format.

604    The categorical class file format (cls) was created for each dataset to define phenotype

605    labels. GSEA was performed using default settings except for "Permutation type" set

606    to "gene_set".

**Single-cell RNA sequencing (scRNA-seq) data analysis**

608    *scRNA-seq, data subset and integration.* Publicly available, scRNA-seq QC-

609    filtered (Level 4) discovery (DIS) and validation (VAL) epithelial datasets were

610    downloaded from HTAN data portal: https://data.humantumoratlas.org/ (HTAN

611    Vanderbilt)[6]. To subset cells of interest, datasets were loaded into Python (v3.9.1) as

612    AnnData objects (v0.7.6) [71]. Both datasets contained metadata attributes, such as

613    polyp type and assigned cell type. This information was used to subset cells annotated

614    as SSL (sessile serrated lesion) and NL (normal colonic biopsy) with the *isin* function

615    of the pandas (v1.3.1) package (Reback et al., 2021). In total, 64,382 NL (VAL: 34,008,

616    DIS: 30,374) and 12,986 SSL (VAL: 6,892, DIS: 6,094) cells were subsetted for

617    downstream analysis. Subsets of cells were loaded into R (v3.6.1) to perform

618    integration with Seurat (v3.2.3) R package (Stuart et al., 2019). Prior to integration,

619    datasets were independently log-normalized with the *NormalisationData* function.

620    2,000 features being highly variable for each dataset and across datasets were

621    identified using *FindVariableFeatures* and *SelectingIntegrationFeatures* functions,

622    respectively. A *FindIntegrationAnchors* function with default settings was implemented

623    to perform the unsupervised identification of cells that were used to integrate DIS and

624    VAL datasets together by applying *IntegrateData* function. Further bioinformatics

625    analysis was performed in Seurat (v4.1.1)[72].

626     *scRNA-seq, dimensional reduction and clustering.* Principal component

627     analysis (PCA) was performed on normalized and scaled data by implementing

628     *RunPCA* function with a features argument set to use variable features. The first 10

629     dimensions of the PCA were used to calculate the neighbors with *FindNeighbors*

630     function. Finally, the graph was segmented into clusters using *FindCluster* function

631     with a resolution adjusted to 0.5. Uniform Manifold Approximation and Projection

632     (UMAP), a non-linear dimensional reduction method, was selected to explore an

633     integrated dataset by implementing *RunUMAP* function with a number of dimensions

634     specified as 10. *DimPlot* function was further used to visualize cells in a low

635     dimensional UMAP space.

636     *scRNA-seq, cell type annotation.* The original cell type annotation, which was

637     included in the metadata, was projected in the UMAP space with *DimPlot* function and

638     used to rename 39 identified clusters by implementing *RenameIdents* function. In total,

639     8 cell types were found within an analyzed subset of SSL and NL cells.

640     *scRNA-seq, gene signature scores, UCell.* UCell (v1.3.1) R package[33] was

641     applied to evaluate cholesterol homeostasis signature distribution in SSL and NL

642     dataset. UCell calculates the gene signature score in individual cells based on the

643     Mann-Whitney U statistic. List of genes involved in cholesterol homeostasis were

644     retrieved from MsigDB[67]. UCell score for cholesterol signature was calculated by

645     implementing *AddModuleScore_UCell* function on Seurat object. Obtained UCell

646     score distribution for SSL and NL dataset was visualized in UMAP space by

647     implementing *FeaturePlot* function. Additionally, a violin plot was generated with

648     *VlnPlot* function to compare cholesterol signature distribution in different cell

649     populations. For statistical analysis, mean comparison p-values were added to violin

650     plots with *stat_compare_means* function from ggpubr (v0.4.0) R package

651    (Kassambara, 2020). Means were compared using the non-parameteric Wilcoxon

652    rank sum test with SSC cluster as a reference group.

653    *scRNA-seq, differential expression testing.* The differential testing of

654    expression of cholesterol biosynthesis genes between SSC and remaining cell

655    populations was performed in Seurat. *FindMarkers* function was run with the 'ident.1'

656    argument defining SSC and 'feature' argument specifying a list of cholesterol genes

657    (74 genes) to test. As a result, 21 genes of cholesterol signature were identified as up-

658    (17 genes) and downregulated (4 genes) in SSC population. Additionally, the average

659    expression of genes upregulated in SSC was visualized across all cell types with

660    *DotPlot* function.

661    *scRNA-seq, gene regulatory network.* Single-Cell rEgulatory Network

662    Inference and Clustering (SCENIC) method was applied to infer gene regulatory

663    network in different cell types of SSL and NL dataset. The required input files included

664    scRNAseq expression matrix, which was generated from Seurat object using

665    build_loom function of ScopeLoomR R package. List of human TFs (1839 genes) and

666    three hg19 gene-motifs ranking databases comparing 10 species in .feather format

667    were downloaded from https://resources.aertslab.org/cistarget/. First, the candidate

668    regulatory modules of TFs and target genes were inferred by using the GRNBoost2

669    method (arboreto, v0.1.6). Next, RcisTarget was implemented to remove indirect

670    targets from these modules based on motif discovery. Finally, the activity of predicted

671    regulons was quantified at the cellular resolution by implementing the AUCell

672    algorithm. Further, resulting regulon activity enrichment matrix was jointly analyzed

673    with count-based matrix by using Scanpy (v1.9.1) python package[71]. To identify

674    regulons that are specific to the SSC cluster we computed the regulon specificity score

675    (RSS)[35]. The RSS was calculated for each cell type with *regulon_specificity_scores*

676  function of pySCENIC and the top 30 regulons for each cell type were shown on plot.

677  Regulon activity of selected regulons was visualized on UMAP transcriptomic space

678  with *sc.pl.umap* function of scanpy package.

679  scRNAse, *regulation detection of deregulated cholesterol genes.* A Seurat's

680  table of twenty-one deregulated cholesterol genes in SSC was imported to Cytoscape

681  (v 3.9.1)[73], where iRegulon (v1.3)[36] was run with default settings to predict TFs

682  potentially regulating the expression of these genes. TFs that were shown previously

683  to be specific for SSC were selected from the combination of motifs and tracks output

684  (cut off > 5 target genes). Generated regulatory network was visualized in Cytoscape

685  with target genes node colored by avg_log2FC.

**Statistical analysis**

687  Statistical significance testing was performed using GraphPad Prism 7

688  software. Comparisons between two groups were performed using the two-tailed,

689  unpaired Student's t-test. Comparisons between more than two groups were

690  performed using ANOVA with appropriate post-hoc analysis for multiple comparison

691  (Tukey, Dunnet). Distribution data for intestinal crypts were analyzed using the

692  Kolmogorov-Smirnov test.

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927 **Conflict of interest.** The authors declare no conflict of interest.

944 **Author contributions.**

945 A.R. and C.A.P designed the experiments. PR and RPG performed bioinformatics

946 analysis, S.W., P.F., H.C., I.G., S.U., L.S. G.C., E.P., H.J., F.H. and S.G. performed

947 the experiments. N.B.S. supported microarray and RNAseq experiments. N.S.A. and

948 A.S-I. performed electron microscopy. A.R, C.A.P, K.B., C.T. and R.F. analyzed the

949 data. AR and PR wrote the manuscript. All authors reviewed the manuscript.

950

951    **Figure legends**

952    **Figure 1. Persistence of ISCs and crypt hyperplasia in *Braf^V600E^* intestinal tissue.**

953    **A)** Schematic of the experimental work: *VillinCre^ER/0^/Braf^V600E-LSL/+^* were culled 3 days

954    and 6 months post induction of *Braf^V600E^* for tissue histology and transcriptomic

955    analysis. Mice were also culled at an intermediate time point 6 weeks post induction

956    to corroborate changes in tissue histology. **B)** Violin plots showing the distribution of

957    number of cells per crypt in control mice and mice induced with *Braf^V600E^* for 3 days

958    (BVE). Dotted and plain lines indicate median and quartiles respectively. Data were

959    analyzed by Kolmogorov-Smirnov test. n=6 mice per group in 3 days, 4 WT and 3

960    mutant mice in 6 weeks, n=4 mice per group in 6 months. 50 crypts were counted per

961    each animal. ****P ≤ 0.0001. **C)** H&E representative images of hyperplastic crypts and

962    quantification of proliferative cells (BrdU+) 3 days post tamoxifen injection. **D)** Violin

963    plots showing the distribution of number of cells per crypt in mice induced for 6 weeks

964    and 6 months plotted and analyzed as in A). ****P ≤ 0.0001. **E)** Histological images of

965    small intestinal tissue stained with the stem cell marker Olfm4 from WT control mice

966    and mice with the *Braf^V600E^* mutation (BVE) at 3 days, 6 weeks and 6 months following

967    tamoxifen induction. Bar size = 100 μm

968    **Figure 2. *Braf^V600E^* expression in the intestinal epithelium alters intestinal**

969    **homeostasis and affects absorptive and secretory cell lines. A)** GSEA showing

970    changes in differentiation gene signatures 3 days and 6 months following tamoxifen

971    induction. NES = normalized enrichment score. **B)** GSEA showing changes in an

972    enterocyte gene signatures 6 months following tamoxifen induction. NES = normalized

973    enrichment score. **C)** Histological images of small intestinal tissue from WT control

974    mice and mice with the *Braf^V600E^* mutation (BVE) 6 months following tamoxifen

975    induction stained with alkaline phosphate (Alpl) to assess enterocyte cells. **D)** TEM

976     images of WT and *Braf* mutant intestinal tissues showing altered microvilli in mutant

977     mice. scale bar = 1 μm. **E)** Quantification of Goblet and ChgA+ enteroendocrine cells

978     from the indicated mice. Each dot represents a single mouse and lines represent mean

979     ± SEM. Data were analyzed by unpaired two-tailed t-test (n=3 per group). *P ≤ 0.05,

980     **P ≤ 0.001. **F)** rt-qPCR analysis of the expression of the intestinal biomarkers

981     Chromogranin A, *(ChgA*, EECs) 6 months after induction of *Braf$^{V600E}$*. Data are plotted

982     as mean ± SD and were analyzed by unpaired two-tailed t-test (n=4 per group). *P ≤

983     0.05.

984     **Figure 3. Expression of *Braf$^{V600E}$* in the intestinal epithelium induces persistent**

985     **gene signatures related to CRC. A)** GSEA showing changes in signatures

986     associated with *BRAF$^{V600E}$*-driven CRC (WT vs *Braf$^{V600E}$*) 3 days and 6 months

987     following tamoxifen induction. NES = normalized enrichment score. **B)** GSEA showing

988     changes in the Wnt signatures 3 days and 6 months following tamoxifen induction.

989     NES = normalized enrichment score. **C)** GSEA showing enrichment in a poor-

990     prognosis gene signature for *BRAF$^{V600E}$*-drive CRC 6 months following tamoxifen

991     induction. NES = normalized enrichment score. **D)** GSEA showing dependence on

992     MAPK pathway for the establishment of key gene signatures. Mice expressing

993     *Braf$^{V600E}$* for 3 days were treated with vehicle control or the MEK inhibitor PD184352

994     (MEKi). NES = normalized enrichment score.

995     **Figure 4. *Braf$^{V600E}$* in the intestinal epithelium shows enriched expression of a**

996     **gene signature related to cholesterol metabolism. A)** GSEA showing changes in

997     gene expression of the cholesterol biosynthesis signature 3 days and 6 months

998     following tamoxifen induction. NES = normalized enrichment score. **B)** Treatment of

999     mice with the MEK inhibitor PD184352 reverts the enrichment of the cholesterol

1000     biosynthesis gene signature in intestinal tissue. NES = normalized enrichment score.

1001 **C)** rt-qPCR analysis of cholesterol metabolism genes in intestinal tissue collected 6

1002 months after tamoxifen injection. Data are reported as fold over WT control mice. Bars

1003 represent mean ± SEM. Data were analyzed by unpaired two-tailed t-test (n=4 per

1004 group). **P ≤ 0.01; ***P ≤ 0.001. **D)** GSEA showing increased transcriptional

1005 expression of cholesterol biosynthesis gene signature in the intestinal tissue of two

1006 independent *Braf*$^{V600E}$ mouse datasets. NES = normalized enrichment score**. E)** Violin

1007 plot showing the distribution of number of cells per crypt in *Braf* mutant mice induced

1008 for 3 days and treated with vehicle control or atorvastatin 10 mg/mL. Dotted and plain

1009 lines indicate median and quartiles respectively. Data were analyzed by Kolmogorov-

1010 Smirnov test. n=6 mice in the vehicle group and 3 mice in the atorvastatin-treated

1011 group. 50 crypts were counted per each animal **P ≤ 0.01. **F)** and **G)** Cell proliferation

1012 and apoptosis were evaluated in *Braf* mutant intestinal crypts of mice treated with

1013 vehicle control or atorvastatin using IHC for BrdU and cleaved-PARP1, respectively.

1014 Examples of cleaved-PARP positive crypt cells are provided. Data are plotted as box

1015 and whiskers plots. Whiskers represent min and max value. Data were analyzed using

1016 unpaired, two-tailed t-test (n=6 vehicle and 3 atorvastatin treated animals). Ns = no

1017 significant ****P ≤ 0.0001.

1018 **Figure 5. Gene signatures of cholesterol biosynthesis are enriched in**

1019 **transcriptomic datasets of human colorectal neoplasia. A)** and **B)** GSEA showing

1020 increased expression of the cholesterol gene signature in human SSLs from 3

1021 independent datasets comparing SSLs to normal matched control (A) or to

1022 adenomatous lesions (B). **C)** UMAP visualization of cells from SSL and NL dataset

1023 colored by annotated cell type. ABS, absorptive cells; CT, crypt top colonocytes; EE,

1024 enteroendocrine cells; GOB, goblet cells; SSC, serrated specific cells; STM, stem

1025 cells; TAC, transient amplifying cells; TUF, tuft cells  **D)** UCell score distribution for the

1026 cholesterol gene signature shown in UMAP space. **E)** Violin plot showing a

1027 comparison of UCell score distribution of a cholesterol gene signature in different cell

1028 populations. Distribution of UCell score in SSC population was compared with

1029 remaining cell lineages using Wilcoxon statistical test. ****P ≤ 0.0001.

1030 **Figure 6. A)** Regulon activity projected in UMAP space for the regulons associated

1031 with transcription factors (FOSL1, SP6, KLF16, NR2F1, CEBPB, CREB3) involved in

1032 the establishment of the cholesterol gene signature in the SSCs. **B)** A network diagram

1033 generated with iRegulon in Cytoscape using the differentially expressed gene of the

1034 cholesterol biosynthesis signature in SSC identified by Seurat as an input.  Circles

1035 indicate 21 significant genes, colored by avg_Log2FC. Octagons symbolizes selected

1036 TFs (cut off > 5 target genes) involved in the regulation of DEGs in the cholesterol

1037 biosynthesis signature. The edges represent connections between each of six TFs

1038 and their target genes, and are colored based on the source of integration.

1039 **Supplemental Figure legends**

1040 **Supplemental Figure 1. Expression of *Braf^V600E* in the intestinal epithelium**

1041 **reduces animal lifespan and generates limited tumorigenesis. A)** Kaplan-Meyer

1042 survival analysis showing reduced survival of *Braf^V600E* mice (median survival 622 days

1043 and 414 days, P<0.0001 Mantel-Cox Log-rank test). **B)** Graph showing the number of

1044 tumors in mice culled at the indicated time post-tamoxifen injection. **C)** H&E

1045 representative images of intestinal tissue hyperplasia (HP) and intestinal lesions.

1046 LGD=low grade dysplasia, HGD=high grade dysplasia, Ca=cancer.

1047 **Supplemental Figure 2. Transcriptomic analysis of mice after short term (3 days)**

1048 **and long-term (6 months) induction of the *Braf^V600E* oncogene. A)** Principal

1049 component analysis (PCA) of *Braf^V600E* mice and WT controls showing clustering by

1050    genotype. n=6 animals per genotype. **B)** Volcano plot of differentially expressed genes

1051    in *Braf^V600E* versus WT control mice. Log2 for fold change in expression on the X-axis

1052    and Log10 for adjust P value on Y-axis. Results are color coded: adjusted p value less

1053    than 0.05 (grey), log2 fold change greater than 0.5 (green), adjusted p value less than

1054    0.05 (blue), and both adjusted p value less than 0.05 and log2 fold change greater

1055    than 0.5 (red). **C)** Heat map with the most top significantly up and down regulated

1056    genes in *Braf^V600E* intestine.

1057    **Supplemental Figure 3.** GSEA showing persistent enrichment of a MAPK signature

1058    in the intestinal tissue of *Braf^V600E* mice. NES = normalized enrichment score.

1059    **Supplemental Figure 4. Persistence of ISCs following expression of *Braf^V600E* in**

1060    **intestinal tissue. A)** rt-qPCR analysis of intestinal stem cells biomarkers *Lgr5* and

1061    *Olfm4* 6 months after tamoxifen injection. Each dot represent a single mouse and lines

1062    represent mean ± SEM. Data were analyzed by unpaired two-tailed t-test (n=4 per

1063    group). ns = not significant. **B)** GSEA 3 days and 6 months after induction of mutant

1064    *Braf* showing changes in the expression of an intestinal stem cells signature. NES =

1065    normalized enrichment score.

1066    **Supplemental Figure 5. Expression of *Braf^V600E* in the intestinal epithelium alters**

1067    **homeostasis of Paneth cells. A)** Representative histological images of small

1068    intestinal tissue stained with the lysozyme marker from WT mice and *Braf^V600E* mice

1069    (BVE) at 3 days, 6 weeks and 6 months following tamoxifen induction. Bar size = 100

1070    μm. **B)** Details of delocalized Paneth cells in 6-month induced mutant mice. Bar size

1071    = 100 μm (20 μm inset). C) rt-qPCR analysis of the expression of the Paneth cell

1072    marker Lysozyme 6 months after induction of *Braf^V600E*. Data are plotted as mean ±

1073    SD and were analyzed by unpaired two-tailed t-test (n=4 per group). *P ≤ 0.05.

**Supplemental Figure 6. Expression of *Braf^V600E* in the intestinal epithelium alters intestinal tissue homeostasis. A)** Representative histological images of small intestinal tissue from WT mice and *Braf^V600E* mice (BVE) at 3 days, 6 weeks and 6 months following tamoxifen induction. Bar size = 100 $\mu$m. **B)** Bar graph showing quantification of villi length in mice 3 days, 6 weeks and 6 months following tamoxifen induction. Data are plotted as mean ± SEM. Data were analyzed by unpaired two-tailed t-test (n=3 per group). ***P ≤ 0.001, ****P ≤ 0.0001.

**Supplemental Figure 7. Expression of *Braf^V600E* in the intestinal epithelium alters homeostasis of EE cells.** Representative histological images of small intestinal tissue stained with Alcian Blue (Goblet cells) and the EEC marker ChgA in the intestinal tissue of mice with the indicated genotypes. Independent WT mice were used for each different time point, but, for ease of reference, representative examples from 6 months mice are shown. Bar size = 100 $\mu$m.

**Supplemental Figure 8. GSEA of mouse transcriptomic data. A)** and **B)** most significant up and down regulated signatures from the MSigDB in *Braf^V600E* mice at 3 days (A) and 6 months (B) following induction of mutant *Braf*.

**Supplemental Figure 9. Analysis of the TCGA transcriptomic dataset.** GSEA analysis shows significant enrichment of the Myc targets, E2F targets and G2M checkpoint signatures. NES = normalized enrichment score.

**Supplemental Figure 10. Inhibition of MAPK pathway with the MEK inhibitor PD1843352. A)** Schematic of the experimental design for *in vivo* inhibition of the MAPK pathway using the pharmacological inhibitor PD184352. **B)** Western blot analysis of phosphorylated Erk protein in intestinal tissue showing efficient inhibition of the MAPK pathway in mice treated with the MEK inhibitor PD184352 (MEKi). **C)**

1098 Principal component analysis (PCA) of $Braf^{V600E}$ mice treated with MEKi or vehicle.

1099 n=6 animals per genotype. **D)** Volcano plot of differentially expressed genes in

1100 $Braf^{V600E}$ treated with MEKi versus vehicle control mice. Log2 for fold change in

1101 expression on the X-axis and Log10 for adjust P value on Y-axis. Results are color

1102 coded: adjusted p value less than 0.05 (grey), log2 fold change greater than 0.5

1103 (green), adjusted p value less than 0.05 (blue), and both adjusted p value less than

1104 0.05 and log2 fold change greater than 0.5 (red). **E)** Heat map with the most top

1105 significantly up and down regulated genes in $Braf^{V600E}$ mice treated with MEKi. **F)**

1106 GSEA showing downregulation of MAPK pathway signature in mice expressing

1107 $Braf^{V600E}$ for 3 days and treated with the MEK MEKi. NES = normalized enrichment

1108 score.

1109 **Supplemental Figure 11. Expression of $Braf^{V600E}$ in the mouse intestinal**

1110 **epithelium leads to enrichment of a fetal-like gene signature. A)** GSEA of the fetal

1111 and metaplasia gene signatures in two independent human dataset comparing SSLs

1112 to tubular adenomas or matched normal tissue. **B)** GSEA of the fetal signature the

1113 intestinal tissue of $Braf^{V600E}$ mice 3 days and 6 months following induction of mutant

1114 *Braf*. Dependence on MAPK is also shown. NES = normalized enrichment score.

1115 **Supplemental Figure 12. Expression of metaplastic genes *Aqp5* and *Anxa10* in**

1116 **intestinal tissue of mice with the $Braf^{V600E}$ mutation.** Normalized expression levels

1117 of the *Aqp5* and *Anxa10* genes in the intestinal tissue at the indicated time points.

1118 Dependence on MEKi is also shown. P value was determined using Wald test. Data

1119 are plotted as box and whiskers plots. The bar within the box represent the median

1120 value, whiskers represent min and max value.

1121 **Supplemental Figure 13. Analysis of human transcriptomic datasets. A)** GSEA

1122 shows lack of transcriptional enrichment of the cholesterol gene signature in

1123 adenomas lesions compared to match normal tissue.

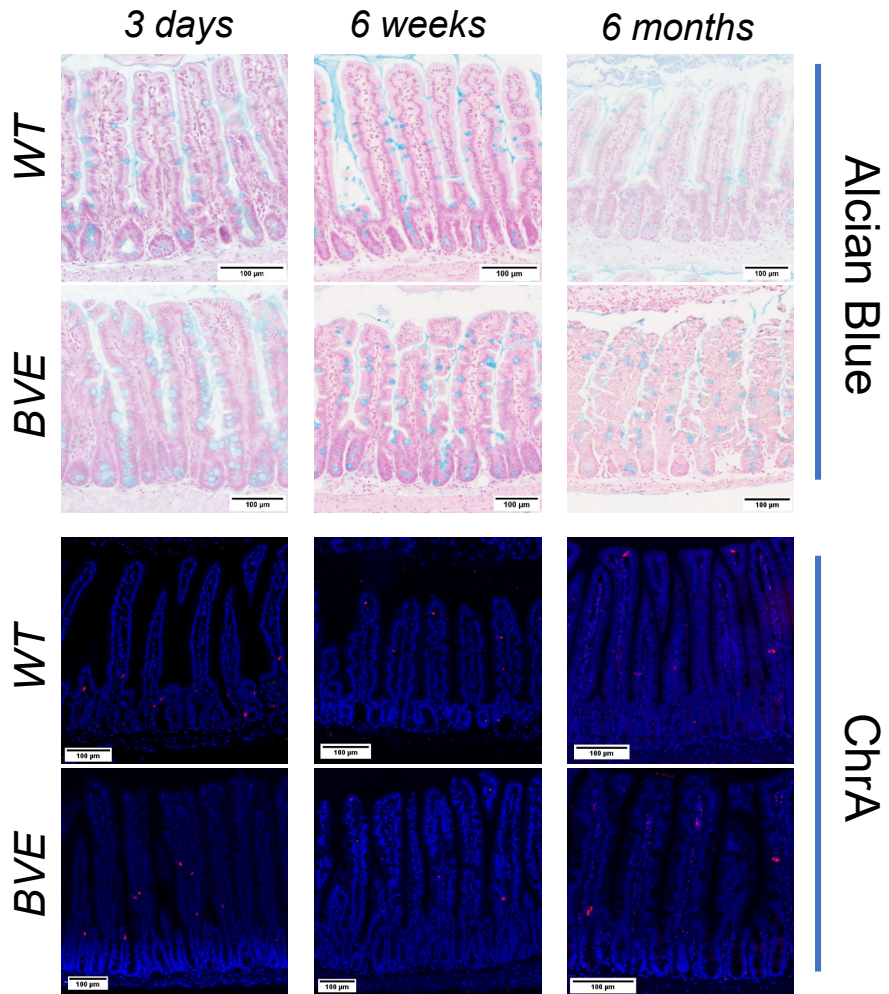1124 **Supplemental Figure 14. Single cells analysis of cholesterol biosynthesis in**

1125 **SSLs. A)** UMAP representation of epithelial scRNA-seq data, color-coded by cell type

1126 and comparing cell population in, normal tissue (NL), SSL, sessile serrated lesions.

1127 ABS, absorptive cells; CT, crypt top colonocytes; EE, enteroendocrine cells; GOB,

1128 goblet cells; SSC, serrated specific cells; STM, stem cells; TAC, transient amplifying

1129 cells; TUF, tuft cells. **B)** Dot plot of genes within the cholesterol signature showing

1130 significant upregulation in gene expression between SSC and non-tumor cells. Dot

1131 color indicates average gene expression levels, dot size indicates the percentage of

1132 cells within a population that express the indicated gene.

1133 **Supplemental Figure 15. Analysis of regulons in SSLs.** Plot of the top 30 specific

1134 regulons (highlighted in red) in the indicated cell lines from analysis of single cell

1135 data of SSL and NL dataset. The regulon specificity score (RSS) is shown on y axis.

1136 ABS, absorptive cells; CT, crypt top colonocytes; EE, enteroendocrine cells; GOB,

1137 goblet cells; SSC, serrated specific cells; STM, stem cells; TAC, transient amplifying

1138 cells; TUF, tuft cells.

1139 **Supplemental Figure 16. The effects of cholesterol biosynthesis inhibition on**

1140 **cell viability in *BRAF*$^{V600E}$ colorectal cancer cells.** The effect of different

1141 concentrations (0, 5 and 10 $\mu$M) of atorvastatin and periods of treatment (24, 48 and

1142 72 hrs) on RKO (A) and HT-29 (B) cell lines. Values were normalized against the

1143 control mean by dividing the measured optical density by the mean control optical

1144 density and multiplying by 100 to give cell viability as a percentage of the control.

1145     The error bars represent standard error of the mean (SEM). Results were obtained

1146     from two independent experiments. Significance levels were measured against the

1147     controls for each treatment period and between treatment concentrations for each

1148     treatment period using a two-way ANOVA and Tuckey post hoc analysis *$p<0.05$,

1149     **$p<0.01$, ***$p<0.005$, ****$p<0.001$.

1150

*Figure 1*
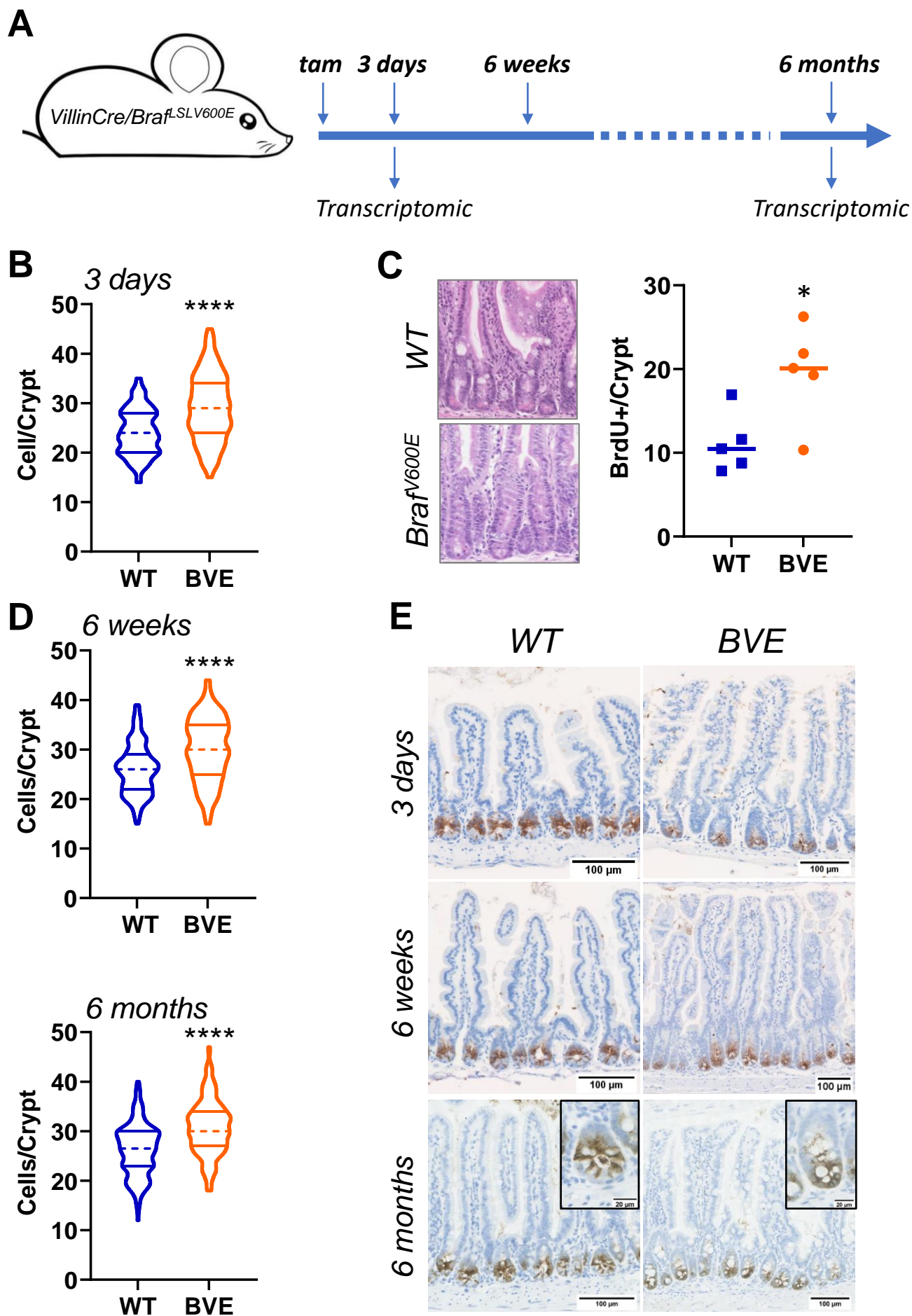
**Figure 2**

*Figure 3*

*Figure 4*

**A**

*3 days*

Cholesterol homeostasis

NES = 2.8
adjP= 1E-10

*BVE*  *WT*

Rank in Ordered Dataset

*6 months*

Cholesterol

NES = 2.9
adjP= 1E-10

*BVE*  *WT*

Rank in Ordered Dataset

**B**

*BVE + MEKi vs BVE*

Cholesterol

NES = -2.48
adjP < 0.0001

*MEKi*  *vehicle*

Rank in Ordered Dataset

**C**

Fold over WT control

**

***

***

HMGCR  MVK  MVD

**D**

*Tong et al. BrafV600E*

Enrichment plot:
HALLMARK_CHOLESTEROL_HOMEOSTASIS

NES = 2.4
adjP = 0.0

'BRAF' (positively correlated)

Zero cross at 6195

'CONTROL' (negatively correlated)

Rank in Ordered Dataset

— Enrichment profile — Hits — Ranking metric scores

*Leach et al. Braf^{V600E};Tgfbr1^{-/-}*

Enrichment plot:
HALLMARK_CHOLESTEROL_HOMEOSTASIS

NES = 2
adjP = 0.0

'BRAF_DAY30' (positively correlated)

Zero cross at 7813

'CTRL_DAY30' (negatively correlated)

Rank in Ordered Dataset

— Enrichment profile — Hits — Ranking metric scores

**E**

Cells/Crypt

**

Vehicle  Statin

**F**

BrdU+ cells/crypt

ns

Vehicle  Atorvastatin

**G**

Cleaved-PARP cells/crypt

****

Vehicle  Atorvastatin

*Vehicle*

*Statins*

**Figure 5**

Figure 6

**A**



**B**

**A**



**B**



**C**

3 days



6 months

**A**

### Lgr5



### Olfm4



**B**

### 3 days



NES= -1.34
Padj= 0.013

### 6 months



NES=1.28
Pajd=0.045

**A**



**B**



**C**

**A**



**B**

**A**



3d BRAF V600E - Hallmark pathways GSEA

**B**



6m V600E - Hallmark pathways GSEA

**Enrichment plot: HALLMARK_MYC_TARGETS_V1**

NES= 1.8
Padj= 0.044

**Enrichment plot: HALLMARK_E2F_TARGETS**

NES=1.9
Padj= 0.03

**Enrichment plot: HALLMARK_G2M_CHECKPOINT**

NES= 2
Padj= 0.008

**A**

*VillinCRE^{ER};Braf^{LSL-V600E/+}*

Day 1    Day 2    Day 3    Day 4    Day 5

Transcriptomic

*tam*    *tam*    *MEK i*    *MEK i*    *MEK i*

**B**

| | WT | | Cre | | BVE | |
| PD184352 | - | + | - | + | - | + |

pERK1/2

ERK2

**C**



**D**

3d

Differential expression

**E**



**F**

MAPK

NES = -2.2
adjP= 3.9E-6

*MEKi*    *vehicle*

**A**

*De Sousa et al. SSL vs Tubular*   *Parker et al. SSL vs Normal*

**B**

*3 days*   *6 months*   *MEK inhibition*

## Acquaporin 5

## Annexin 10

**3 days**

Normalized Expression of Aqp5 (3d)

Normalized Expression of Anxa10 (3d)

**6 months**

Normalized Expression of Aqp5 (6m)

Normalized Expression of Anxa10 (6m)

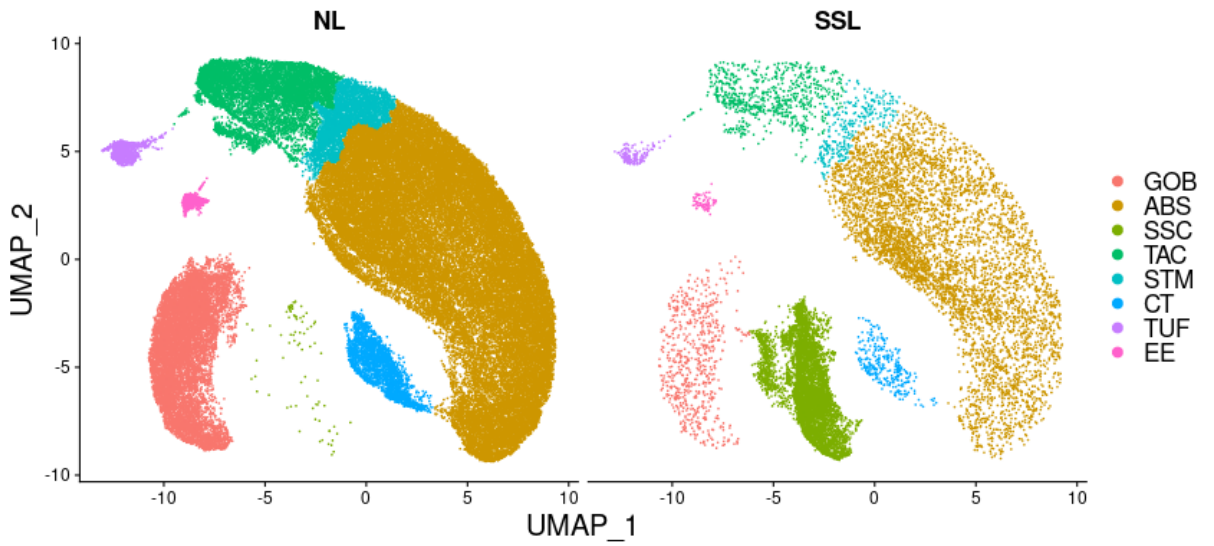**3 days + MEKi**

Normalized Expression of Aqp5 (MEKi)
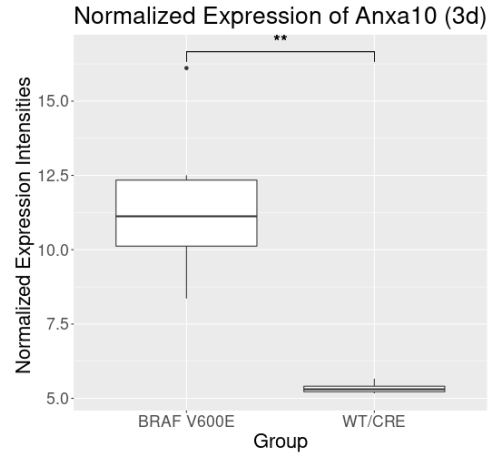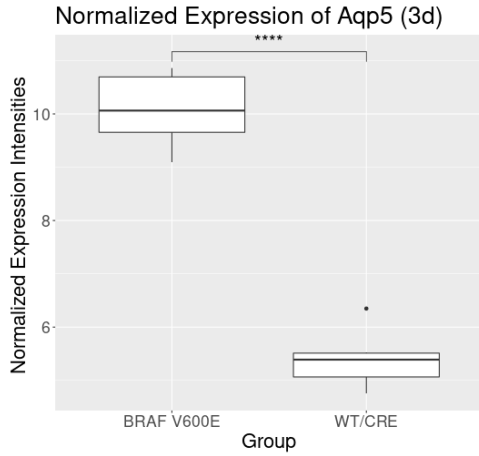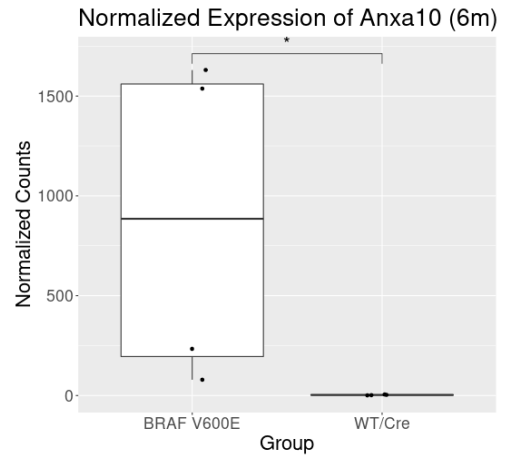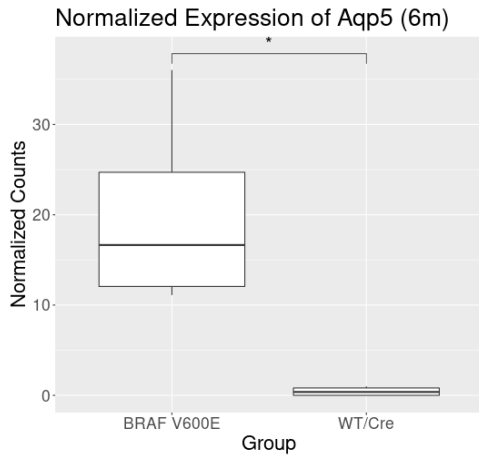
Normalized Expression of Anxa10 (MEKi)
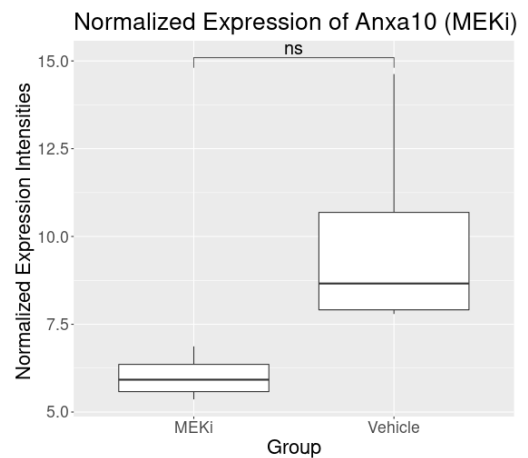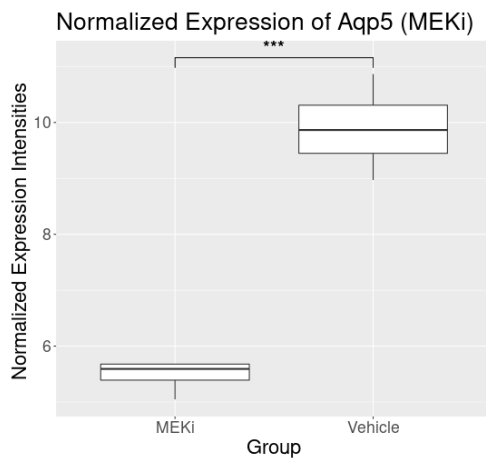
# <u>ADs vs normal colon</u>
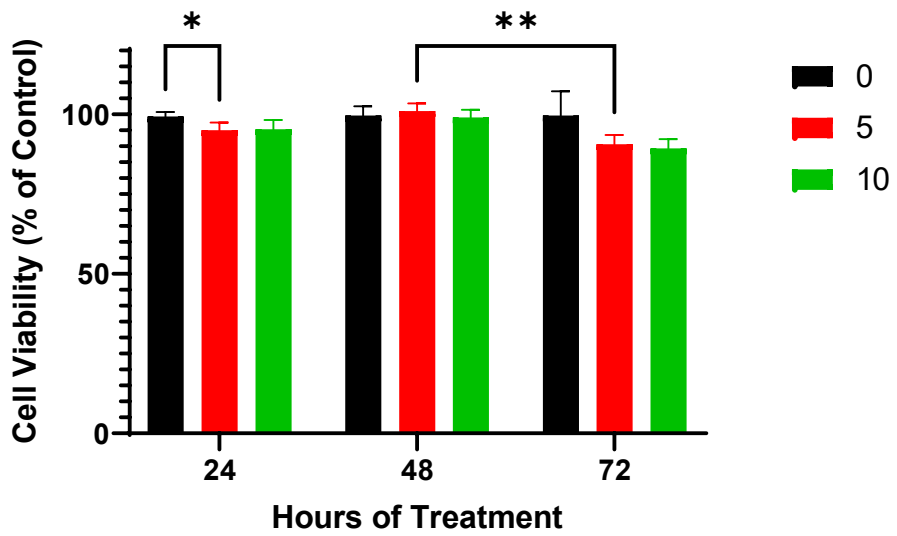
*Parker et al.*
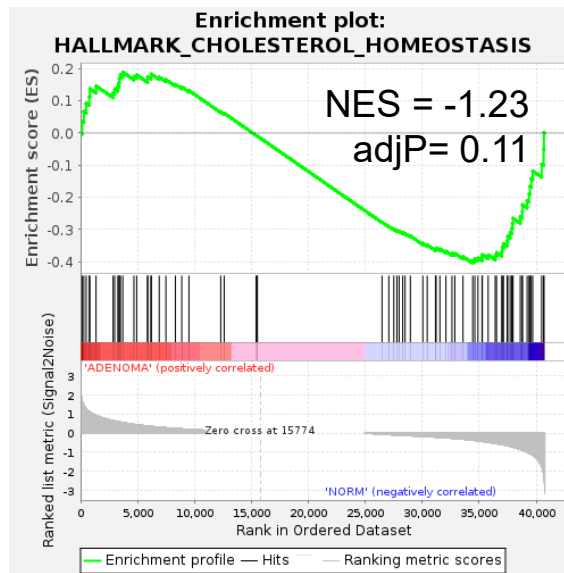
**A**
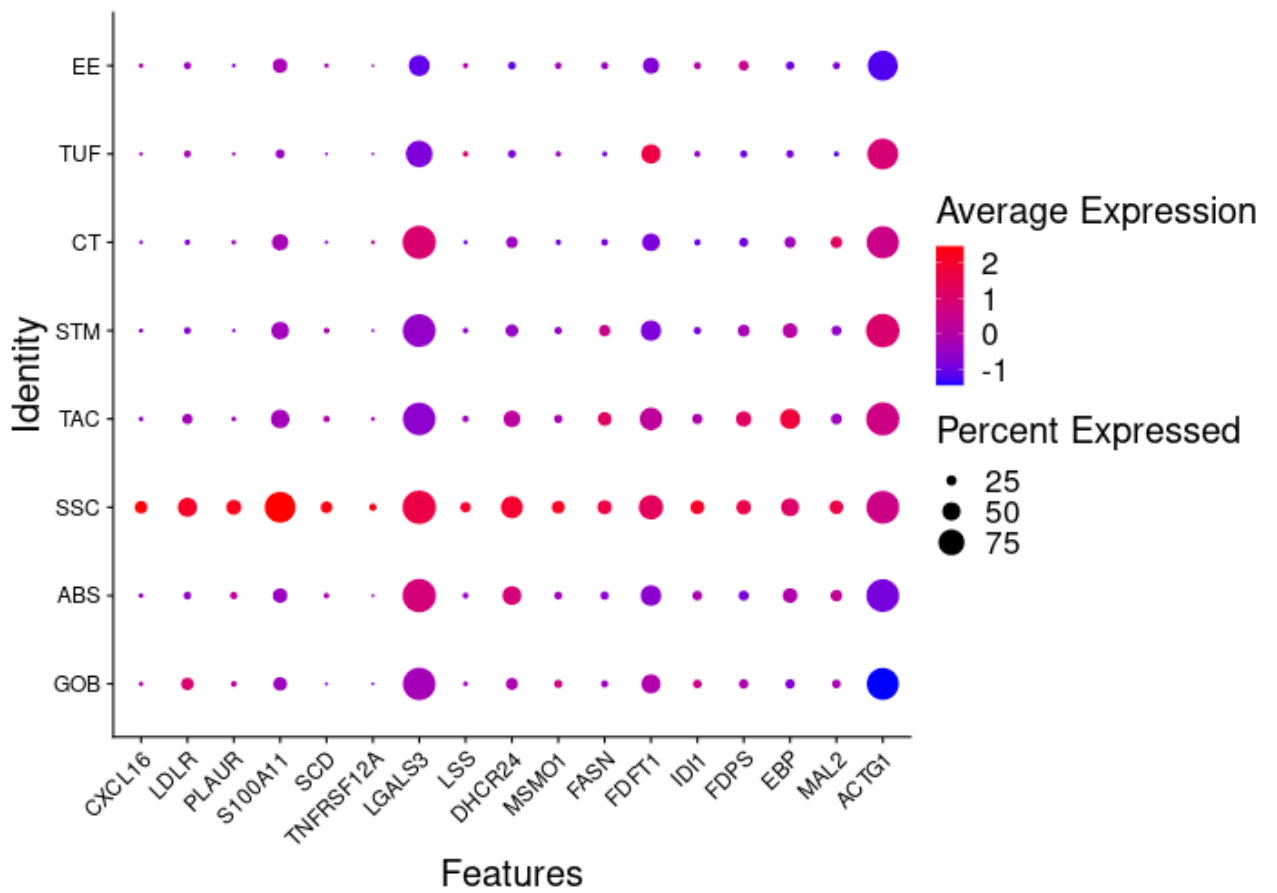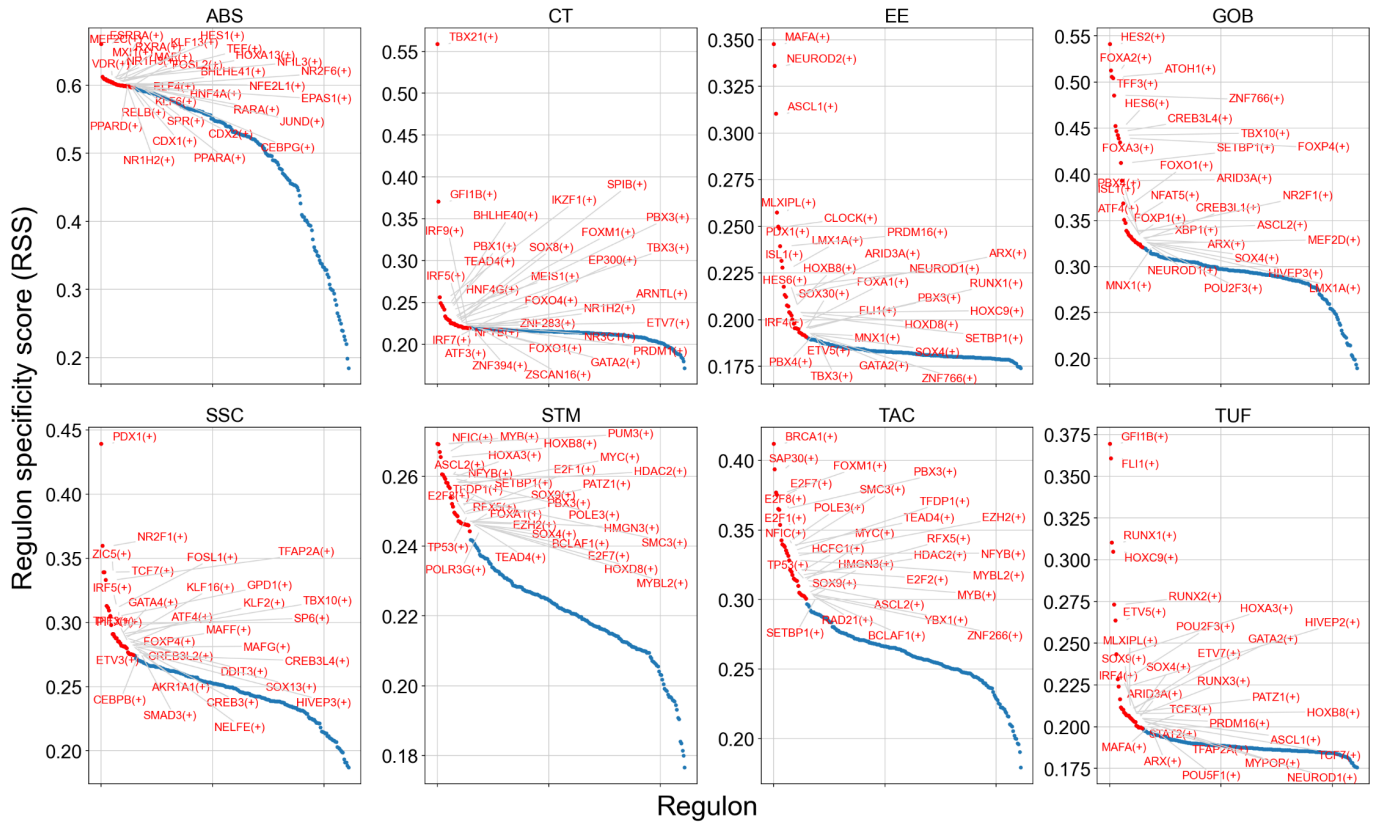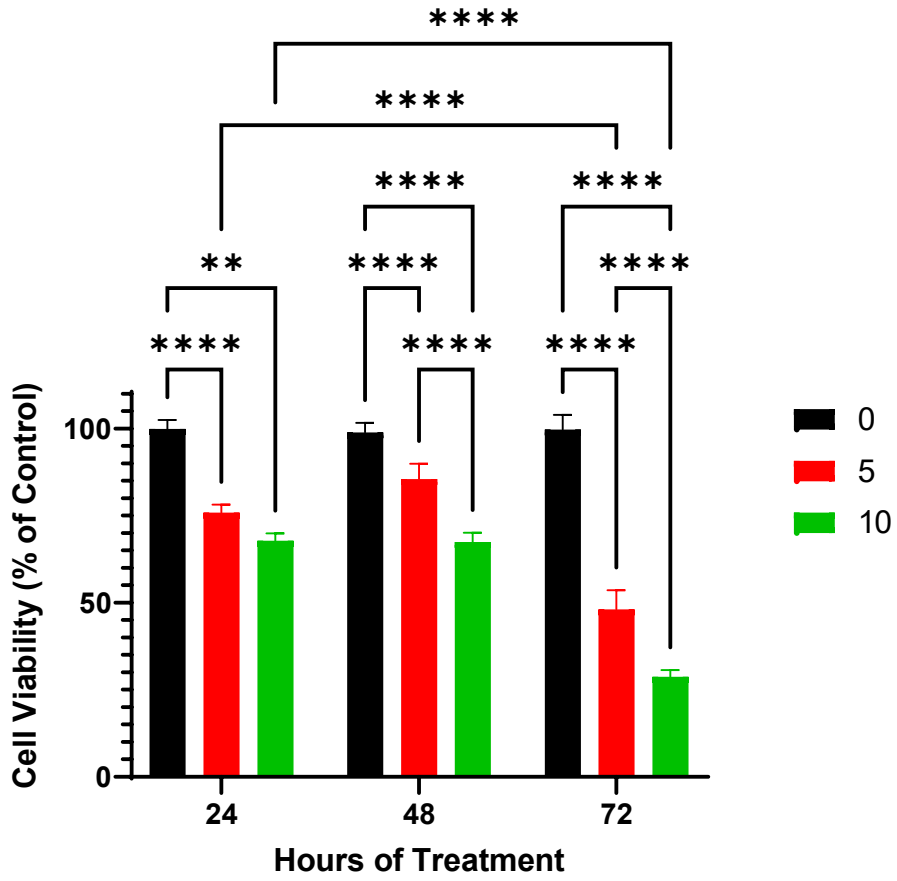


**B**

| Target | Primers | Sequence |
|---|---|---|
| **BRAFLSL-V600E/WT** | OCP125 (FWD) | 5'-GCC-CAG-GCT-CTT-TAT-GAG-AA-3' |
| | OCP137 (REV-HET) | 5'-GCT-TGG-CTG-GAC-GTA-AAC-TC-3' |
| | OCP 143 (REV-WT) | 5'-AGT-CAA-TCA-TCC-ACA-GAG-ACC-T-3' |
| **Villin-CreER$^T$** | OCP 361 (FWD) | 5'-GCC-TGG-TCT-GGA-CAC-ATG-CC-3' |
| | OCP362 (REV) | 5'-GTG-TCA-GCA-TCC-AAC-AAG-GC-3' |

**Supplemental Table 1.** PCR primers used for genotyping.

| Target | Supplier | Catalogue | Species | Dilution | Antigen retrieval |
|--------|----------|-----------|---------|----------|-------------------|
| Lysozyme | DAKO | A099 | Rabbit | 1:10,000 | Proteinase K |
| Olfm4 | Cell Signalling | 39141 | Rabbit | 1:300 | Sodium citrate |
| Chromogranin A | Immunostar | 20085 | | 1:1000 | Sodium Citrate |
| BrdU | Cell Signalling | 5292 | Mouse | 1:200 | Sodium Citrate |
| Cleaved-PARP | Cell Signalling | 94885 | Rabbit (mono) | 1:100 | Sodium Citrate |

**Supplemental Table 2.** List of primary antibodies used for IHC and IF.

| Name | Fluorophore | Amplicon size (bp) | Assay ID |
|---|---|---|---|
| Lgr5 | FAM | 64 | Mm00438890_m1 |
| Olfm4 | FAM | 130 | Mm01320260_m1 |
| Lyz1 | FAM | 129 | Mm00657323_m1 |
| Alpi | FAM | 60 | Mm01285814_g1 |
| Muc2 | FAM | 66 | Mm01276696_m1 |
| Chga | FAM | 59 | Mm00514341_m1 |
| Hmgcr | FAM | 64 | Mm01282499_m1 |
| Mvk | FAM | 67 | Mm00445773_m1 |
| Mvd | FAM | 109 | Mm00507014_m1 |
| Tbp | VIC | 65 | Mm01277042_m1 |
| Actb | VIC | 72 | Mm04394036_g1 |
| B2m | VIC | 77 | Mm00437762_m1 |
| Gusb | VIC | 71 | Mm01197698_m1 |

**Supplemental Table 3.** List of TaqMan essay for rt-qPCR.